

# Real-time PCR quantification and live-cell imaging of endophytic colonization of barley (*Hordeum vulgare*) roots by *Fusarium equiseti* and *Pochonia chlamydosporia*

Jose G. Maciá-Vicente<sup>1</sup>, Hans-Börje Jansson<sup>1</sup>, Nicholas J. Talbot<sup>2</sup> and Luis V. Lopez-Llorca<sup>1</sup>

<sup>1</sup>Laboratory of Plant Pathology, Department of Marine Sciences and Applied Biology, Multidisciplinary Institute for Environmental Studies (MIES) Ramón Margalef, University of Alicante, Apto 99, 03080 Alicante, Spain; <sup>2</sup>School of Biosciences, Geoffrey Pope Building, University of Exeter, Exeter EX4 4QD, UK

## Summary

Author for correspondence:

Jose G. Maciá-Vicente  
Tel: +34 96 5903400 ext 3280  
Fax: +34 96 5909897  
Email: jgm@ua.es

Received: 17 September 2008  
Accepted: 22 November 2008

© New Phytologist (2009)  
doi: 10.1111/j.1469-8137.2008.02743.x

**Key words:** *Agrobacterium tumefaciens*-mediated transformation, *Fusarium equiseti*, green fluorescent protein, *Hordeum vulgare* (barley), live cell imaging, *Pochonia chlamydosporia*, real-time PCR, root endophytes.

- New tools were developed for the study of the endophytic development of the fungal species *Fusarium equiseti* and *Pochonia chlamydosporia* in barley (*Hordeum vulgare*) roots. These were applied to monitor the host colonization patterns of these potential candidates for biocontrol of root pathogens.
- Molecular beacons specific for either *F. equiseti* or *P. chlamydosporia* were designed and used in real-time polymerase chain reaction (PCR) quantification of fungal populations in roots. Genetic transformation of isolates with the green fluorescent protein (*GFP*) gene was carried out using an *Agrobacterium tumefaciens*-mediated transformation protocol, and spatial patterns of root colonization were investigated by laser confocal microscopy.
- Quantification of endophytes by real-time PCR in roots of barley gave similar results for all fungi, and was more accurate than culturing methods. Conversely, monitoring of root colonization by *GFP*-expressing transformants showed differences in the endophytic behaviours of the two species, and provided evidence of a plant response against endophyte colonization.
- Both *F. equiseti* and *P. chlamydosporia* colonized barley roots endophytically, escaping attempts by the host to prevent fungal growth within root tissues. This strongly supports a balanced antagonism between the virulence of the colonizing endophyte and the plant defence response. Development of real-time PCR techniques and *GFP* transformants of these fungal species will facilitate future work to determine their biocontrol capacity.

## Introduction

*Fusarium equiseti* and *Pochonia chlamydosporia* (formerly *Verticillium chlamydosporium*) are two soil-inhabiting fungal species that colonize plant roots. *Fusarium equiseti* has been characterized as a natural root endophyte (Maciá-Vicente *et al.*, 2008a) with the capacity to colonize plant roots and with properties that could make it a promising candidate for biological control of root pathogens and nematodes (Nitao *et al.*, 2001; Horinouchi *et al.*, 2007; Maciá-Vicente *et al.*, 2008b). *Fusarium equiseti* has also, however, been described as a plant pathogen with potential to control parasitic plants such as *Striga hermonthica* (Kirk, 1993). *Pochonia chlamydosporia* is a parasite of nematode eggs with a wide occurrence in cyst and root-knot nematode-infested soils

around the world (Kerry, 1993). However, *P. chlamydosporia* has also been found to parasitize phytopathogenic fungi (Jacobs *et al.*, 2003; Monfort *et al.*, 2005) and to colonize barley (*Hordeum vulgare*) and tomato (*Solanum lycopersicum*) roots endophytically under laboratory conditions (Bordallo *et al.*, 2002; Lopez-Llorca *et al.*, 2002). Furthermore, colonization of roots by this fungus may promote growth of the host plant (Monfort *et al.*, 2005; Siddiqui & Akhtar, 2008). When considered together, these properties make *P. chlamydosporia* a potential biocontrol agent for both disease-causing fungi and plant-parasitic nematodes.

Endophytism, the capacity of an organism to colonize tissues of a host plant without causing disease symptoms, is a complex process influenced by several physiological and environmental factors. The effective establishment of a plant–

fungal endophyte relationship has frequently been shown to produce benefits for the plant, such as alleviation of biotic and abiotic stresses, or growth promotion (Schulz & Boyle, 2005, and references therein). However, the capacity of a fungus to develop within the plant tissues also provides it with an increased fitness due to ease of finding nutrients, suitable environmental conditions, and avoidance of the microbial antagonism it would experience outside of the plant. Thus, root endophytism would provide a stable source of inoculum to sustain the populations of a potential biocontrol organism in the rhizospheric soil, where it is intended that it should carry out its biocontrol activity.

Recent investigations using culturing techniques in our laboratory have revealed that both *F. equiseti* and *P. chlamydosporia* are able to colonize barley roots and persist for long periods as stable endophytes in potted plants, conferring benefits on their hosts (J. G. Maciá-Vicente *et al.*, unpublished). Nevertheless, the study of fungal colonization of roots using classical cultivation techniques under nonaxenic conditions is difficult and, in many cases, does not provide a good descriptor of the rhizosphere competence of a given fungus. A high frequency of isolation (or re-isolation, after an artificial infection of the host plant) of a fungal species does not necessarily correlate with a high degree of colonization, and vice versa (Schulz & Boyle, 2005). Other classical approaches used to study root colonization, such as conventional microscopy techniques, also have limitations. Therefore, newer, more efficient methodologies need to be optimized for the study of root endophytes in order to determine their efficacy as biocontrol agents. Real-time polymerase chain reaction (PCR) is currently the most accurate method for quantifying fungal colonization of host tissues (Schena *et al.*, 2004; Schulz & Boyle, 2005). This methodology has successfully been employed for this purpose, mostly to detect or quantify phytopathogenic fungi (Winton *et al.*, 2002; Gao *et al.*, 2004; Schena *et al.*, 2004), but also mycorrhizas (Gamper *et al.*, 2008; Hortal *et al.*, 2008) and other endophytes (Deshmukh *et al.*, 2006). Green fluorescent protein (GFP) tagging of genetically transformed fungal species is a breakthrough in microscopical detection of endophytes within host tissues (Bloemberg & Camacho-Carvajal, 2006) because it allows live-cell imaging of fungal colonization, enabling the spatial and temporal dynamics of fungal colonization to be studied. The recent development of *Agrobacterium tumefaciens*-based transformation protocols for filamentous fungi provides increased transformation yields and stability of transformants compared with other transformation methods, and has increased the number of successfully transformed fungal species (de Groot *et al.*, 1998; Rho *et al.*, 2001; Sesma & Osbourn, 2004; Sugui *et al.*, 2005; Fang *et al.*, 2006).

In the present work, we have adapted the above-mentioned methodologies for detection and quantification of *F. equiseti* and *P. chlamydosporia* during their development as endo-

phytes in barley roots. Primers and molecular beacons (Tyagi & Kramer, 1996), based on the translation elongation factor 1 $\alpha$  (*tef-1 $\alpha$* ) gene for *F. equiseti*, and on the alkaline serine protease p1 (*vcp1*) gene for *P. chlamydosporia*, were designed and used for detection of these fungi in the barley rhizosphere. Both *tef-1 $\alpha$*  and *vcp1* have been described as single-copy genes (Morton *et al.*, 2003; Geiser *et al.*, 2004), and this may improve the accuracy of the quantification of fungal cells using real-time PCR. *Agrobacterium tumefaciens*-mediated transformation with the *GFP* gene of each species was also performed to study the barley root colonization patterns of these fungi. These techniques will be useful for future research on the endophytic behaviour of these biological control agents. Here we present evidence of stable endophytic colonization by *F. equiseti* and *P. chlamydosporia* and critically evaluate these methods for studying the endophytic and biocontrol potential of these fungal species.

## Materials and Methods

### Fungal isolates, bacterial strains and plant material

*Fusarium equiseti* (Corda) Saccardo isolates 10/3.3.1 (Fe10331) and 45/1.2.1 (Fe45121), and *Pochonia chlamydosporia* (Goddard) Zare & Gams isolates 123 (Pc123) and INEM-VC-21 (Pc21) were used for real-time PCR probe design and detection experiments. *Fusarium equiseti* isolate Fe10331 and *P. chlamydosporia* isolate Pc123 were used for *A. tumefaciens*-mediated genetic transformation with the GFP gene.

*Fusarium equiseti* Fe10331 and Fe45121 were isolated from roots of natural vegetation growing on a sandy soil and a salt marsh in southeast Spain, respectively (Maciá-Vicente *et al.*, 2008a). For molecular beacon specificity assays on *F. equiseti*, genomic DNA was extracted from the following *Fusarium* isolates: *Fusarium avenaceum* subsp. *aywarte* Sangal. & L.W. Burgess 19/1.2 (Fa1912), *F. equiseti* 28/3.2.1 (Fe28321) and 50/1.1.1 (Fe50111), *Fusarium globosum* Rheeder, Marasas & P.E. Nelson 16/1.3.1 (Fg16131), *Fusarium hostae* Geiser & Juba 27/1.3.1 (Fh27131), *Fusarium negundinis* Sherb. 15/2.3.1 (Fn15231), *Fusarium oxysporum* Schlechtend. emend. Snyder & Hans 5.4 (Fo54) and 13.1 (Fo131), *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg 63/1.3.2 (Fp63132), *Fusarium redolens* Wollenw. 60/2.1.1 (Fr60211), and *Fusarium solani* (Mart.) Sacc. 3/1.6.1 (Fs3161) and 30/2.1.1 (Fs30211) (Maciá-Vicente *et al.*, 2008a).

*Pochonia chlamydosporia* Pc123 was isolated from infected *Heterodera avenae* eggs (Lopez-Llorca & Duncan, 1986). Pc21 was a kind gift from Dr A. Ciancio (Istituto per la Protezione delle Piante, Consiglio Nazionale delle Ricerche, Bari, Italy). The specificity of *P. chlamydosporia* molecular beacons was tested using genomic DNA of the isolates *Pochonia rubescens* Zare, W. Gams & Lopez-Llorca 10 (Pr10), *Lecanicillium dimorphum* (J.D. Chen) Zare & W. Gams 139 (Ld139),

**Table 1** Primers and molecular beacon probes used for quantification of fungal colonization of barley (*Hordeum vulgare*) roots by real-time PCR

Fungus	Primers/probe	Sequence (5'→3')	Target DNA	Amplicon size (bp)
<i>Fusarium equiseti</i>	EFEQ-F <sup>1</sup>	AGGTTGGTTTCCATTTTC		
	EFEQ-R <sup>2</sup>	CGAGTAGCGGGGTATA		
	MBFEQ <sup>3</sup>	FAM-ccgcgggACTGAATATGCGCCTGTTACccccgag-TAMRA	<i>tef-1α</i>	154
<i>Pochonia chlamydosporia</i>	SPC-F <sup>1</sup>	CGTTTCCCAGGACTACAAGA		
	SPC-R <sup>2</sup>	CGGCAACTGAGAGGAAGA		
	MBPC <sup>3</sup>	FAM-ctcgcccGGTGCCATTGCTTCCATGAGTCgggcgag-TAMRA	<i>vcp1</i>	136

<sup>1</sup>Forward primer. <sup>2</sup>Reverse primer. <sup>3</sup>Molecular beacon probe. GC-rich stems are shown in lowercase letters. *tef-1α*, translation elongation factor 1α; *vcp1*, alkaline serine protease p1.

and *Lecanicillium cf. psalliotae* (Treschow) Zare & W. Gams 197 (Lp197).

The fungi tested, from the culture collection of the Laboratory of Plant Pathology (University of Alicante, Alicante, Spain), were grown at 22°C on corn meal agar (CMA; BBL, Sparks, MD, USA). DNA extractions from fungal mycelium were performed as described in O'Donnell *et al.* (1998), with slight modifications.

*Agrobacterium tumefaciens* AGL-1 (Lazo *et al.*, 1991) strains including the vector pCAMBGFP (Sesma & Osbourn, 2004) or pFBENGFP (Fang *et al.*, 2006) were used for Fe10331 and Pc123 genetic transformation, respectively.

Barley (*Hordeum vulgare* L. var. *disticum*) was used as the host plant for root inoculation experiments.

#### Detection of *F. equiseti* and *P. chlamydosporia* endophytes by real-time PCR

**Design of molecular beacons** To design primers and molecular beacon probes for detection of *F. equiseti* by real-time PCR, 87 *Fusarium tef-1α* gene region sequences (GenBank accession numbers DQ854847–DQ854941) obtained by Maciá-Vicente *et al.* (2008a) were aligned using the CLUSTAL W multiple alignment tool (Thompson *et al.*, 1994) and optimized manually. Forward and reverse primers to amplify a 154-bp fragment of the gene and an internal 19-bp sequence were designed in conserved regions for *F. equiseti*.

*Pochonia chlamydosporia* primers and probe design were based on the *vcp1* gene. The complete sequence of this gene for isolate Pc123 was first obtained. Primers VCP-1F (5'-GAACTCCTCATCCCGCTGATT-3') and VCP-1R (5'-GAAACTCTGGTAGATTCTTAT-3') were used to amplify, by PCR, a 2260-bp fragment containing the *vcp1* gene, using the following temperature cycles: an initial denaturation step at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s each, annealing at 55°C for 20 s, and extension at 72°C for 3 min, and a final extension step at 72°C for 5 min. Amplification reactions were performed in a total volume of 40 µl containing: 1× Flexi buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.5 µM of each primer, 100 ng of DNA template and 2.5 units of *Taq* DNA polymerase (Promega

Corp., Madison, WI, USA). The amplification product was loaded onto a 0.8% agarose gel stained with SYBR Green I (Sigma, St Louis, MO, USA) and viewed under ultraviolet light. The PCR product was purified using QIAquickPCR (Qiagen, Crawley, UK) columns and sequenced using both VCP-1F and VCP-1R primers, and the internal primers VCP-3F (5'-TGCAACTGTCTGTTCTTCTC-3') and VCP-3R (5'-CCAAGTAGAGAGAATGGCAC-3'). Generated sequences were edited and a contig sequence constructed using the BioEdit Sequence Alignment Editor (Hall, 1999). The *vcp1* sequence from *P. chlamydosporia* Pc123 was entered in GenBank with the accession number FJ009628. This sequence was used as a query in the BLAST tool at GenBank, and the resulting database matched sequences (including other *P. chlamydosporia vcp1* sequences) were included in the analysis. Sequences were aligned using the CLUSTAL W multiple alignment tool and optimized manually. Conserved regions for *P. chlamydosporia* only were selected for primer design defining a 136-bp region, and an internal 23-bp sequence with complete homology to *P. chlamydosporia* sequences was selected to construct a molecular beacon probe.

Two complementary 7-bp long-arm GC-rich sequences were added to either side of each probe sequence. Fluorogenic probes were labelled at their 5' ends with the fluorescent reporter dye fluorescein (FAM), and the 3' end was modified with the quencher dye tetramethyl-6-carboxyrhodamine (TAMRA) (Sigma-Genosys, Saint Quentin Fallavier, France). Primer and probe sequences are described in Table 1.

**Specificity of primers and probes** The specificity of primers for *F. equiseti* based on *tef-1α* was checked by conventional PCR using genomic DNA from isolates Fe10331, Fe45121, Fa1912, Fe28321, Fe50111, Fg16131, Fh27131, Fn15231, Fo54, Fo131, Fp63132, Fr60211, Fs3161 and Fs30211. Amplification reactions were performed in a total volume of 25 µl containing: 1× Flexi buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.5 µM of each of the primers EFEQ-F and EFEQ-R, 10 ng of DNA template and 1 unit of *Taq* DNA polymerase (Promega Corp.). In negative controls, genomic DNA was replaced by water to rule out contamination. Temperature

cycles were carried out in a PTC-100 Thermal cycler (MJ Research, Waltham, MA, USA) with the following steps: an initial denaturation step at 94°C for 5 min and 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 20 s. Positive amplification controls from fungal DNA were performed with primers EF1 and EF2, which generate a *c.* 700-bp product from the *tef-1 $\alpha$*  gene region (Geiser *et al.*, 2004). An aliquot of 4  $\mu$ l of amplification products was separated on a 2% electrophoresis agarose gel stained with SYBR Green I and photographed under ultraviolet light. The specificity of the SPC-F/SPC-R primer set was checked using genomic DNA from isolates Pc123, Pc21, Pr10, Ld139, Lp197, Fe10331 and Fe45121, with the same conditions as for *F. equiseti* primers except that the annealing temperature was 59°C instead of 57°C. In this case, positive amplification controls were performed with the primer set ITS4/ITS1F for the fungal internal transcribed spacer (White *et al.*, 1990; Gardes & Bruns, 1993).

Specificity tests were repeated using real-time PCR for those *Fusarium* isolates that generated the respective 154-bp product with EFEQ-F/EFEQ-R, and for all isolates used for the SPC-1F/SPC-1R primer set. The reaction mix was the same as described in the previous paragraph, but included 0.5  $\mu$ M of the corresponding molecular beacon. Temperature cycles, under the same conditions as described in the previous paragraph, were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), with recording of fluorescence during the annealing step. Each reaction was performed in triplicate. The cycle threshold ( $C_t$ ) value (defined as the cycle number at which a statistically significant increase in the reporter fluorescence can be detected) for each PCR was calculated and analysed using the ABI PRISM SEQUENCE DETECTION software (version 1.2.3). All conventional PCRs and real-time PCRs were performed at least twice with the same results.

**Inoculation of fungi into barley roots for real-time PCR quantification** Barley roots were either inoculated with Fe10331, Fe45121, Pc123 or Pc21, or inoculated with noncolonized CMA plugs (controls) as in Bordallo *et al.* (2002). Briefly, barley seeds were surface-sterilized using 5% NaOCl and germinated for 2 d in germinating medium (Bordallo *et al.*, 2002). Seedlings free from contaminants were placed axenically in 50-ml autoclaved culture tubes (one per tube) with 30 ml of sterile, distilled water-saturated vermiculite. Fungi were inoculated using four 5-mm-diameter CMA plugs from the borders of 2-wk-old colonies, placed 10 mm deep and mixed with the vermiculite. In control treatments, barley roots were inoculated with four noncolonized CMA plugs. Tubes were incubated at 23°C with a photoperiod of 16 : 8 h (light:dark).

Forty barley plants per treatment were sampled 7 d after inoculation (dai) and root systems were separated from the aerial parts of the plants. For each treatment, half of the roots

(from 20 plants) were unsterilized while the remainder were surface-sterilized for 1 min in 1% NaOCl, followed by three rinses (1 min each) in sterilized distilled water and blotted dry onto sterile filter paper. To check that the roots had been effectively surface-sterilized, they were imprinted onto CMA medium to check for fungal propagules on the root surface. Ten plants per treatment were used to assess fungal root colonization by culturing techniques, and the remaining material was stored at -76°C for subsequent DNA extractions. For each plant, either unsterilized or surface-sterilized roots were cut into *c.* 1-cm-long pieces, and 10 root pieces per plant and treatment were plated on Petri dishes containing CMA. Plates were incubated at 22°C in the dark and examined after 7 d for fungal growth. The percentage of root pieces colonized by each fungus was recorded and statistically analysed using the Kruskal–Wallis rank sum test ( $P = 0.05$ ).

**Root DNA extraction** An aliquot of 1–1.5 g of 7-d-old barley root tissue, either unsterilized or surface-sterilized (three replicates per treatment), was ground in liquid nitrogen and DNA was extracted in 4 ml of CTAB (100 mM Tris-HCl pH 8.4, 1.4 M NaCl, 25 mM EDTA, and 2 % hexadecyl-trimethyl-ammonium bromide) extraction buffer containing 2% low-weight polyvinylpyrrolidone (PVP) at 65°C for 1 h. Extracts were purified in 1 volume of phenol–chloroform–isoamyl alcohol (IAA), 25 : 24 : 1, and then in 1 volume of chloroform–IAA, 24 : 1, and precipitated with 1 volume of isopropanol. DNA pellets were washed twice in 70% ethanol, air-hood-dried and resuspended in 1 $\times$  TNE buffer (10 mM Tris-HCl pH 7.4, 200 mM NaCl, and 1 mM EDTA). Ribonuclease A (Sigma) was added to each treatment and tubes were kept for 30 min at 37°C. Extracts were again purified in phenol–chloroform–IAA and chloroform–IAA and precipitated in 2 volumes of absolute ethanol. Pellets were washed twice in 70% ethanol and allowed to dry, and finally DNA was resuspended in 1 $\times$  TE buffer (10 mM Tris-HCl pH 8, and 1 mM EDTA). The DNA was visualized in 0.8% agarose gels stained with SYBR Green I under ultraviolet light, quantified using Hoechst fluorochrome and calf thymus DNA according to Ausubel *et al.* (2002), and stored at 4°C until use.

**Real-time PCR quantification of *F. equiseti* and *P. chlamydosporia* in roots** Positive amplification of root DNA was tested by conventional PCR with a primer set (which we termed HvUB-F and HvUB-R) which amplifies a *c.* 220-bp product from the barley ubiquitin gene (Deshmukh *et al.*, 2006). Real-time PCRs for quantification of Fe10331, Fe45121, Pc123 and Pc21 in both unsterilized and surface-sterilized 7-d-old barley roots were carried out using the same conditions as described in ‘Specificity of primers and probes’ earlier in this section, with the respective primers and probes for each fungal species. A total amount of 40 ng of root DNA was included as a DNA template in each reaction. For

construction of calibration curves, reaction wells containing 10, 1, 0.1, 0.01 and 0.001 ng of total genomic DNA from *F. equiseti* or *P. chlamydosporia* were included, respectively. Each reaction was performed in triplicate.  $C_t$  values from reactions containing fungal DNA only were correlated with the amount of DNA, and  $C_t$  values obtained for wells containing root DNA were interpolated in calibration curves to calculate the quantity of fungal DNA with respect to the total. Quantification of fungal DNA from root extracts was performed at least twice with the same results. To compare fungal colonization among treatments, a one-way ANOVA was applied assuming  $P < 0.05$ .

### Genetic transformation of endophytes with *GFP*

#### *Agrobacterium tumefaciens*-mediated fungal transformation

Genetic transformations mediated by *A. tumefaciens* were performed as in Rho *et al.* (2001), with modifications. *Agrobacterium tumefaciens* AGL-1 containing either pCAMBGF or pFBENGFP was cultured on Luria Broth (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) supplemented with 100  $\mu\text{g ml}^{-1}$  kanamycin, and incubated at 28°C with shaking at 250 rpm for 16–20 h to reach an optical density ( $\text{OD}_{600}$ ) ~0.15. Bacterial cells in a 200–400- $\mu\text{l}$  aliquot of this culture were harvested and LB medium removed by centrifugation. Cells were subsequently resuspended in 5 ml of *A. tumefaciens* induction medium (AIM) and grown for an additional 16–20 h to reach an  $\text{OD}_{600}$  ~0.5–0.6 before mixing with an equal volume of a conidial suspension of the respective fungus ( $5 \times 10^5$  conidia  $\text{ml}^{-1}$ ). The bacteria-conidia mix was plated onto nitrocellulose filters (0.45  $\mu\text{m}$  pore size and 45 mm diameter; Whatman, Kent, UK) on modified solid AIM medium in the presence or absence of 200  $\mu\text{M}$  acetosyringone (AS, Fluka, St Gallen, Switzerland). Co-cultivation plates were incubated at 24°C for a minimum of 48 h and then nitrocellulose filters were transferred to selection plates containing potato dextrose agar (PDA; Oxoid, Hampshire, UK) supplemented with 400  $\mu\text{g ml}^{-1}$  cefotaxime, 100  $\mu\text{g ml}^{-1}$  carbenicillin and 60  $\mu\text{g ml}^{-1}$  streptomycin (Sigma) in order to eliminate *A. tumefaciens* cells, and the respective selective agent for fungal transformants (250  $\mu\text{g ml}^{-1}$  hygromycin B (A.G. Scientific, San Diego, CA, USA) for *F. equiseti* and 100  $\mu\text{g ml}^{-1}$  benomyl (Sigma) for *P. chlamydosporia*). Selection plates were incubated at 24°C for colony development, and individual transformants were subcultured onto fresh PDA plates with the respective selective agent. To create mono-conidial cultures, conidia from each transformant were suspended with sterile water and plated onto new PDA medium under selection. *GFP* expression of transformants was assessed by fluorescence microscopy with appropriate filters.

**Characterization of transformants** To determine the mitotic stability of the transformants, they were subcultured onto

PDA for five consecutive generations in the absence of their respective selective agent. They were then tested for resistance on PDA supplemented with either 100  $\mu\text{g ml}^{-1}$  hygromycin B (*F. equiseti*) or 50  $\mu\text{g ml}^{-1}$  benomyl (*P. chlamydosporia*). Stable transformants were used for growth habit characterization on PDA (growth rate and sporulation) and barley root colonization in vermiculite tubes using culture methods described in 'Inoculation of fungi into barley roots for real-time PCR quantification' earlier in this section. Genomic DNA from the mycelium of each transformant was extracted as previously described (O'Donnell *et al.*, 1998) and integration of the T-DNA in the fungal genome was assessed by PCR amplification of the respective *GFP* gene using primers GFP-1 and GFP-2 (Lee *et al.*, 2002). One stable transformant for both Fe10331 (Fe10331gfp) and Pc123 (Pc123gfp) was selected for root inoculation and microscopy experiments.

#### Barley root inoculation and laser scanning confocal microscopy

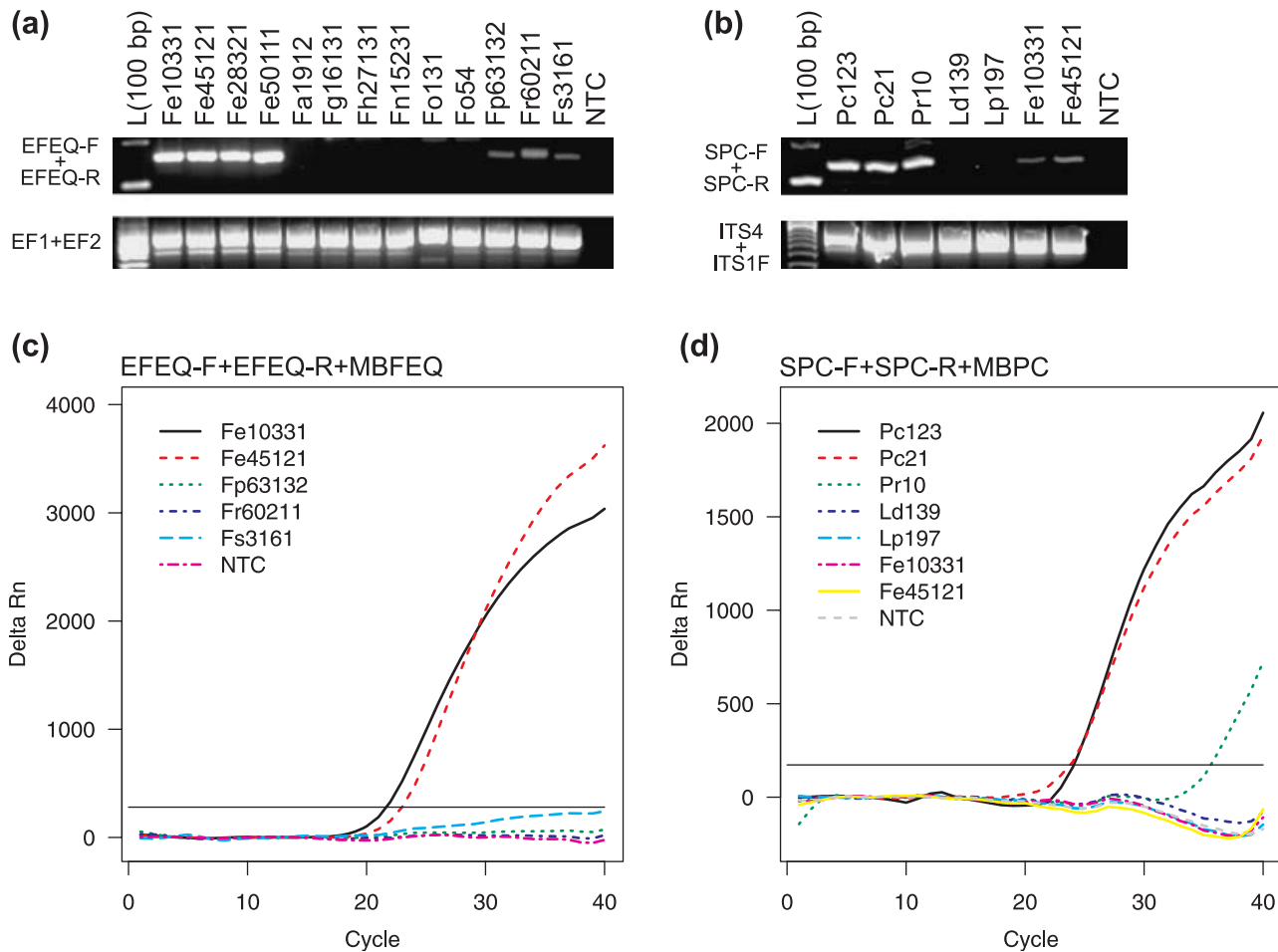
Barley roots were either inoculated with Fe10331gfp or Pc123gfp, or left uninoculated (controls), and plants were grown as described in 'Inoculation of fungi into barley roots for real-time PCR quantification' earlier in this section. Three plants for each treatment were sampled 3, 5 and 7 dai and processed for laser scanning confocal microscopy. Whole roots or longitudinal 50- $\mu\text{m}$ -thick root cryosections (obtained using a Leica CM1510 cryostat; Leica Microsystems, Wetzlar, Germany) were examined in a Leica DM IRBE2 confocal microscope to analyse the dynamics of root colonization by *GFP*-expressing transformants. The *GFP* was excited with a 488-nm laser, and fluorescence was detected at 505–530 nm. Autofluorescence of root cell walls was excited with a 488-nm laser and detected at 580–620 nm.

In a different experiment, inoculated barley roots were sampled 3, 5 and 7 dai, cryosectioned as described in the previous paragraph and stained with the endocytotic lipophilic tracker FM4-64 (Invitrogen, Paisley, UK; catalogue no. 13320) for membrane labelling and detection of nonviable hyphae. Before the observation, root sections were incubated in a 10  $\mu\text{M}$  FM4-64 aqueous working solution for 30 min. Dual labelled samples were simultaneously excited with 488-nm and 543-nm laser lines, and *GFP* emission fluorescence was detected as previously described. FM4-64 emission fluorescence overlapped with the plant cell wall autofluorescence, as they were both detected at 580–620 nm. However, as partial FM4-64 emission fluorescence was also detected at 620–675 nm this wavelength was used to avoid signal overlapping.

## Results

**Real-time PCR quantification of fungal root colonization**

**Specificity of primers and probes** Primers EFEQ-F/EFEQ-R generated a specific 154-bp DNA product for all four



**Fig. 1** Results of specificity assays for primers and probes. (a) Amplification by conventional PCR of DNA extracted from pure cultures of different *Fusarium* species to assess the specificity of EFEQ-F/EFEQ-R primers, showing a specific 154-bp product for *Fusarium equiseti*. Primers EF1/EF2 (Geiser *et al.*, 2004) were used as a positive control for amplification. (b) PCR amplification of DNA extracted from pure cultures of different species to assess the specificity of SPC-F/SPC-R primers, showing the 136-bp product for *Pochonia chlamydosporia* and *Pochonia rubescens*. Primers ITS4/ITS1F (White *et al.*, 1990; Gardes & Bruns, 1993) were used as a positive control for amplification. (c) Real-time PCR amplification and fluorescence readings (Delta Rn) of DNA extracted from pure cultures of *F. equiseti* and other *Fusarium* species which yielded amplification in previous assays, to assess the specificity of MBFEQ. The horizontal solid line indicates the cycle threshold ( $C_t$ ). (d) Real-time PCR amplification and fluorescence readings of DNA extracted from pure cultures of *P. chlamydosporia* and other species included in previous assays, to assess the specificity of MBPC. The horizontal solid line indicates the  $C_t$ . L(100bp), 100-bp ladder; Fe10331, *Fusarium equiseti* 10/3.3.1; Fe45121, *F. equiseti* 45/1.2.1; Fe28321, *F. equiseti* 28/3.2.1; Fe50111, *F. equiseti* 50/1.1.1; Fa1912, *Fusarium avenaceum* subsp. *aywerte* 19/1.2; Fg16131, *Fusarium globosum* 16/1.3.1; Fh27131, *Fusarium hostae* 27/1.3.1; Fn15231, *Fusarium negundis* 15/2.3.1; Fo54, *Fusarium oxysporum* 5.4; Fo131, *F. oxysporum* 13.1; Fp63132, *Fusarium proliferatum* 63/1.3.2; Fr60211, *Fusarium redolens* 60/2.1.1; Fs3161, *Fusarium solani* 3/1.6.1; Fs30211, *F. solani* 30/2.1.1; Pc123, *Pochonia chlamydosporia* 123; Pc21, *P. chlamydosporia* INEM-VC-21; Pr10, *P. rubescens* 10; Ld139, *Lecanicillium dimorphum* 139; Lp197, *Lecanicillium psalliotae* 197; NTC, nontemplate control.

*F. equiseti* isolates included in the assay. Such a 154-bp product was also detected, but at a lower intensity, in isolates *F. proliferatum* Fp63132, *F. redolens* Fr60211 and *F. solani* Fs3161. The primers did not generate the good-sized-amplicon for the rest of the *Fusarium* isolates included in the test (Fig. 1a). Primers SPC-F/SPC-R produced a 136-bp amplicon in the agarose gel for the *P. chlamydosporia* isolates Pc123 and Pc21, and the *P. rubescens* isolate Pr10. The product also appeared as a lower intensity band for both *F. equiseti* isolates, but not for the *Lecanicillium* spp. (Fig. 1b).

Detection of the amplification products of a selection of *Fusarium* isolates (those that generated the specific 154-bp product by conventional PCR) by means of real-time PCR, using the probe MBFEQ, yielded  $C_t$  values of  $21.6 \pm 0.29$  and  $22.9 \pm 0.36$  for Fe10331 and Fe45121, respectively, whereas no significant increase of fluorescence was observed for the other isolates included in the test (Fig. 1c).

Real-time PCR with primers SPC-F and SPC-R and probe MBPC detected both *P. chlamydosporia* isolates, with  $C_t$  values of  $23.7 \pm 0.07$  for Pc123 and  $24.3 \pm 1.1$  for Pc21

(Fig. 1d). Amplification for *P. rubescens* Pr10 was detected at cycle  $35.6 \pm 0.11$  (Fig. 1d), despite the fact that in agarose gels the amplicon intensity was similar to that observed for both *P. chlamydosporia* isolates (Fig. 1b). No PCR products were detected for the remainder of the isolates, including reactions for *F. equiseti* Fe10331 and Fe45121, which had generated a weak amplification product in the conventional PCR assay of approximately 136 bp. The PCR amplification product obtained for Pr10 was purified from the agarose gel and sequenced using both primers SPC-F and SPC-R, to compare its nucleotide sequence with that of *P. chlamydosporia*. The sequence obtained (GenBank accession number FJ009629) revealed that the target sequence for MBPC was completely homologous to that of *P. chlamydosporia* (data not shown).

#### Quantification of *F. equiseti* and *P. chlamydosporia* in roots

Real-time PCR amplification of root extracts with respective primers and probes yielded no significant production of amplicons for control treatments (uninoculated plants), for both unsterilized and surface-sterilized roots (Fig. 2a,b). For treatments in which roots were inoculated with either *F. equiseti* or *P. chlamydosporia*,  $C_t$  values were always achieved earlier for unsterilized roots ( $C_t$  values of 26.2–27.7 for *F. equiseti*, and 27.9–30 for *P. chlamydosporia*) than for surface-sterilized roots ( $C_t$  values of 28.8–30.9 for *F. equiseti*, and 31.2–34.6 for *P. chlamydosporia*), indicating a higher amount of initial target DNA in the former. Amplification of standard DNA for construction of calibration curves was performed as for the root extracts. Adequate correlations of DNA quantity versus  $C_t$  in standard reactions for *F. equiseti* (Fig. 2c) and *P. chlamydosporia* (Fig. 2d) were obtained, demonstrating the accuracy of the PCR-based quantification. We detected quantities as small as 1 pg of *F. equiseti* target DNA, and 10 pg for *P. chlamydosporia*. Quantification of fungal target DNA with respect to the total genomic root DNA showed that overall colonization of the root (unsterilized roots) was statistically similar ( $P < 0.05$ ) for all isolates, with values that ranged from  $5.07 \pm 1.4$  to  $10.59 \pm 2.77$  ng of target DNA per 100 ng of total DNA (Fig. 2e). Colonization of the internal root was also similar ( $P < 0.05$ ) for all *F. equiseti* and *P. chlamydosporia* isolates, with values between  $0.46 \pm 0.05$  and  $1.06 \pm 1.15$  ng of target DNA per 100 ng of total DNA (Fig. 2e). Quantification of root colonization using culturing techniques showed that all unsterilized root fragments were colonized by the respective fungus, and endophytic colonization of roots was also statistically similar ( $P < 0.05$ ) for all isolates tested (Fig. 2f). Colonization ratios of surface-sterilized:unsterilized barley roots were assessed with both techniques. These analyses showed that *P. chlamydosporia* Pc21 yielded the lowest proportion of endophytic growth versus overall growth (0.05 for real-time PCR, and 0.12 for culturing methods), followed by *F. equiseti* Fe10331 (0.07 and 0.13, respectively) (Fig. 2e,f). When colonization was assessed by real-time PCR these ratio

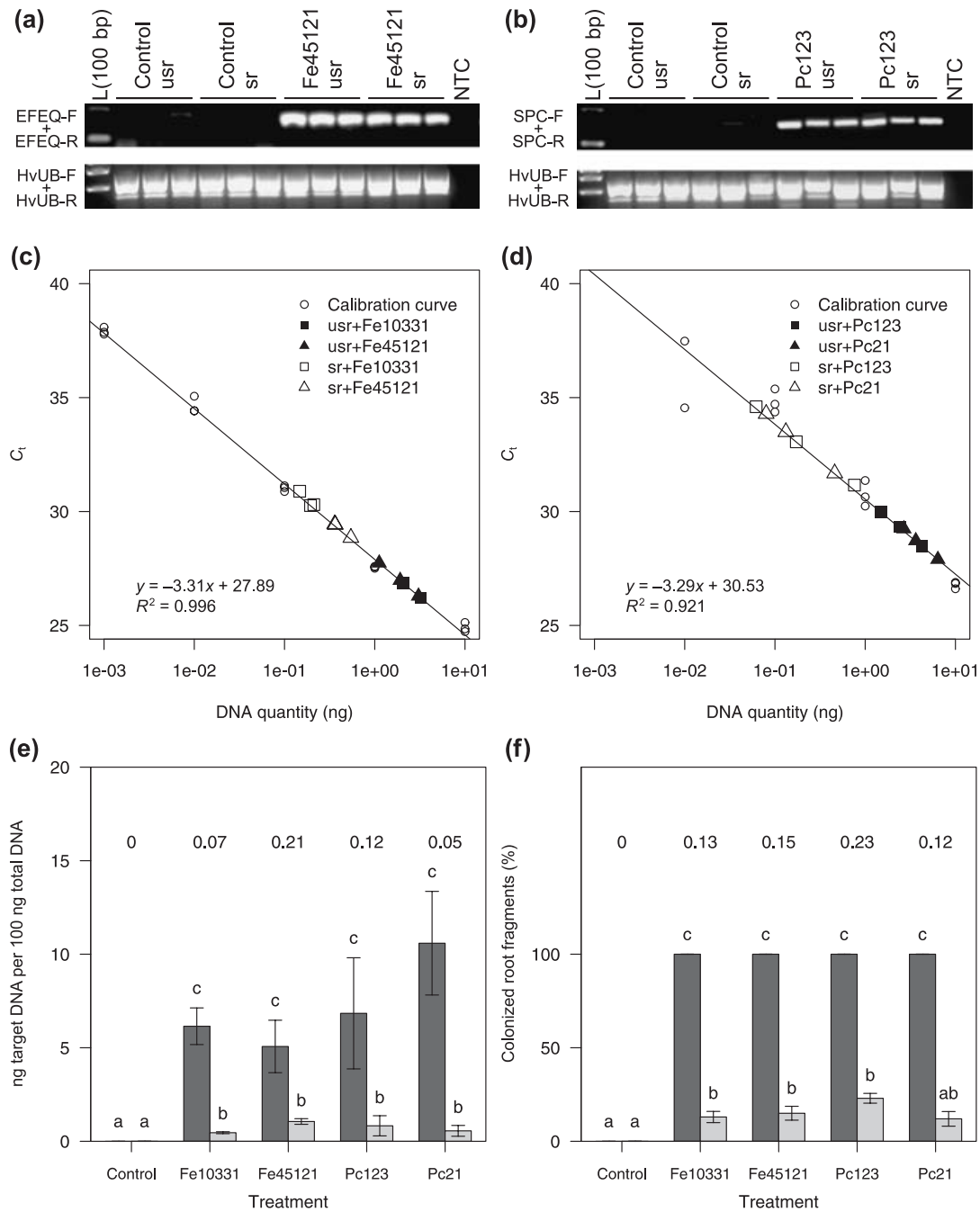
values were lower ( $P < 0.01$ ) for all isolates except *F. equiseti* Fe45121 than when using culturing methods (Fig. 2e,f).

#### Genetic transformation and root colonization by transformants

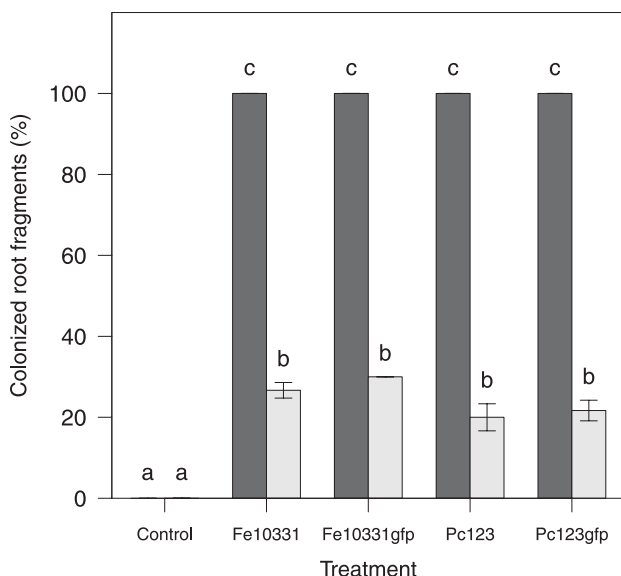
**Characterization of transformants** A total of 26 hygromycin B resistant colonies developed from selection plates for *F. equiseti* Fe10331 transformants. All transformants displayed *GFP* expression when observed with an epifluorescence microscope, in contrast to the wild type (wt). Assessment of mitotic stability revealed that all of the transformants maintained resistance to hygromycin B after five generations in the absence of selection. All transformants presented a growth habit on PDA similar to that of the wt (data not shown). After these assays, two transformants showed intense fluorescence emission when observed by epifluorescence microscopy (Fig. 4a), and one of them (Fe10331gfp) was selected for further study.

Up to 200 colonies developed from selection plates with benomyl for transformed *P. chlamydosporia* Pc123, although few of them showed fluorescence. Therefore, we assessed *GFP* expression in the 30 fastest developing colonies. Only five isolates showed mitotic stability after five generations in the absence of benomyl, and these had a growth habit similar to that of the wt. From these, only one (Pc123gfp) presented intense fluorescence emission under fluorescence microscopy (Fig. 5a), and hence this transformant was selected for barley root inoculation experiments. Integration of the T-DNA in the genomes of both Fe10331gfp and Pc123gfp was confirmed by PCR amplification of *GFP* with the GFP-1/GFP-2 primer set (data not shown). In experiments for characterization of the root colonization ability of the transformants, both GFP-tagged isolates showed a comparable capacity to colonize barley roots to that of the respective wt, as assessed by root plating at 7 dai (Fig. 3). Furthermore, Fe10331gfp and Pc123gfp colonies developing from roots maintained GFP fluorescence when observed by epifluorescence microscopy.

**Laser scanning confocal microscopy of roots** Observation of both *F. equiseti* Fe10331gfp and *P. chlamydosporia* Pc123gfp in barley roots was easily achieved using laser scanning confocal microscopy, as a result of discrimination between GFP fluorescence and autofluorescence of the plant cell walls by use of different detection fluorescence wavelengths. Fe10331gfp rapidly colonized barley roots, forming an extensive hyphal network in their upper part (closest to the fungal inoculum) at 3 dai (Fig. 4b), with penetration of the root epidermis at some points. The density of the outer root mycelium decreased after 5–7 dai (Fig. 4c) and conversely running hyphae within the root cortex became more common. In every case, *F. equiseti* hyphae were always detected within the epidermal cell and outer cortical cell layers, both inter- and



**Fig. 2** Quantification of *Fusarium equiseti* and *Pochonia chlamydosporia* colonization of barley (*Hordeum vulgare*) roots. (a) Example of amplification by conventional PCR with primers EFEQ-F/EFEQ-R of DNA extracted from noninoculated (control) or Fe45121-inoculated barley roots, either unsterilised (usr) or surface-sterilized (sr). Positive amplification controls were performed with primers HvUB-F/HvUB-R (Deshmukh *et al.*, 2006), which amplify a c. 220-bp product from the barley ubiquitin gene. (b) Example of amplification by conventional PCR with primers SPC-F/SPC-R of DNA extracted from noninoculated (control) or Pc123-inoculated barley roots, either unsterilized (usr) or surface-sterilized (sr). Positive amplification controls were performed with primers HvUB-F/HvUB-R. (c) Real-time PCR quantification of *F. equiseti* colonization of barley roots by plotting cycle threshold ( $C_t$ ) values from root extracts against the calibration curve. Each point is the average from three reaction replicates. (d) Real-time PCR quantification of *P. chlamydosporia* colonization of barley roots by plotting  $C_t$  values from root extracts against the calibration curve. Each point is the average from three reaction replicates. (e) Quantification results for unsterilized (black bars) and surface-sterilized (grey bars) barley root colonization by endophytes obtained using real-time PCR. Numbers above bars indicate the endophytic:overall colonization ratio for each treatment. (f) Quantification of unsterilized (black bars) and surface-sterilized (grey bars) barley root colonization by endophytes obtained using culturing methods. Numbers above bars indicate the endophytic:overall colonization ratio for each treatment. L(100 bp), 100-bp ladder; Fe10331, *Fusarium equiseti* 10/3.3.1; Fe45121, *F. equiseti* 45/1.2.1; Pc123, *Pochonia chlamydosporia* 123; Pc21, *P. chlamydosporia* INEM-VC-21; NTC, nontemplate control.



**Fig. 3** Comparison of the isolation percentages from either unsterilized (usr; black bars) or surface-sterilized (sr; grey bars) barley (*Hordeum vulgare*) roots at 7 d after inoculation, between the wild-type Fe10331 and Pc123 strains, and the GFP-tagged transformant for each species (Fe10331gfp and Pc123gfp, respectively).

intracellularly (Fig. 4d) and in root hairs, but never reached the root vascular system. Appressorium-like structures were often observed (Fig. 4e) in running hyphae on plant cell walls between neighbouring epidermal and cortical cells. In most cases the fungus within root tissues consisted of single hyphae crossing root cells, although some cortical cells appeared to be completely filled with hyphae forming vesicles (Fig. 4f) or clusters (Fig. 4g) at 5 dai. At this time, other hyphal swellings, in most cases septate, were formed intercellularly (Fig. 4h). These structures, which resembled chlamydo-spores, became especially abundant at 7 dai.

Root colonization by Pc123gfp started at 3 dai, with discrete hyphal aggregates developing on the root surface which penetrated the epidermal layers (Fig. 5c). These always appeared to be related to root hairs at different root depths (even if they were more common in the upper parts of the roots), with no evident connection between them, and occasionally formed chlamydo-spores on the root surface (Fig. 5h). This first explorative colonization developed with an extensive mycelial coverage of the root surface at 5 and 7 dai (Fig. 5b), with an increase of penetration of the epidermal cells (Fig. 5d). Penetration of the epidermis was often found to be achieved through formation of appressorium-like structures directly on the epidermal cell walls, which elicited papilla formation on plant cell walls to prevent fungal penetration (Fig. 5e). Although at 3 dai fungal colonization of root tissues occurred at very low frequency, generally within the epidermal cell layers (Fig. 5f), this became more common at 5 dai. At this time, colonization of the epidermal and cortical cell layers

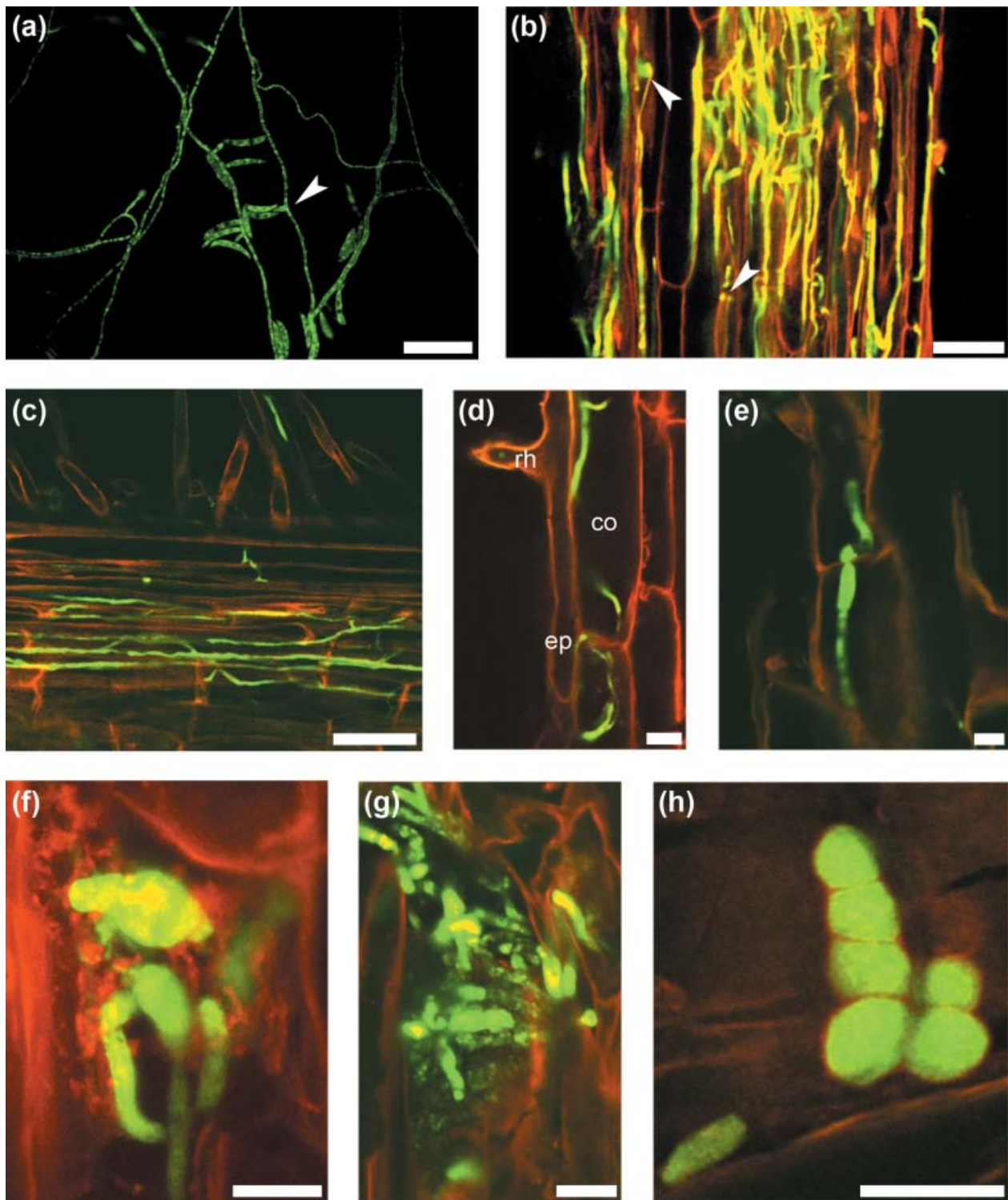
was frequently observed, consisting mostly of intercellular hyphae with formation of appressorium-like structures between adjacent cells. Root colonization reached the inner cortical cells, further than the two to three layers observed for *F. equiseti* (Fig. 5g), with the occasional appearance of intracellular chlamydo-spores (Fig. 5i).

FM4-64 loading studies showed the presence of a membrane sheath covering the invasive hyphae from both *F. equiseti* and *P. chlamydo-sporea* (Fig. 6a,b). This membrane labelling appeared primarily in recently developed hyphae within the plant cell lumen, preventing symplastic fungal growth, but seemed to disappear or become less evident in older hyphae. Some root regions were filled with cellular debris and degraded hyphae, together with other structured nonfluorescent hyphae, which might be nonviable. The completely internalized FM4-64 yielded different emission patterns from those of viable hyphae, which emitted both GFP and FM4-64 fluorescence (Fig. 6c–e). At 7 dai, certain areas of the root contained mostly dead or degraded hyphae (Fig. 6f), with abundant papillae (Fig. 6g) for both fungal species. This was in contrast to other root regions that were more recently colonized, which showed only GFP-expressing hyphae.

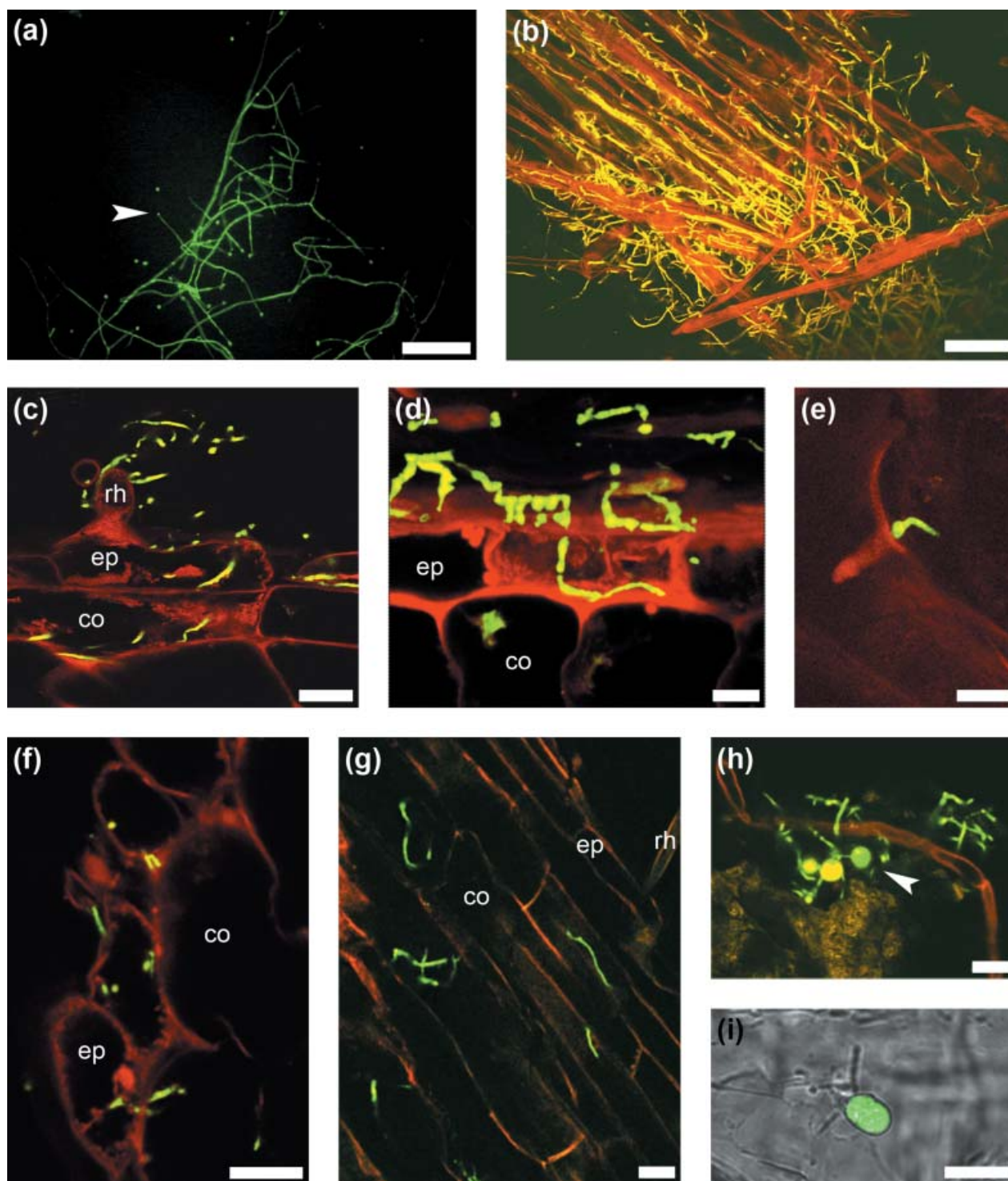
The addition of the FM4-64 dye allowed observation of a large number of vacuolar structures, as a result of the vacuole membrane labelling (Fig. 7). Furthermore, vacuole contents emitted autofluorescence which could be registered at all detection wavelengths. Such vacuoles seemed to be generated as a plant response against fungal colonization of the roots by both *F. equiseti* (Fig. 7c) and *P. chlamydo-sporea* (Fig. 7b,d), as these were absent in uninoculated controls (Fig. 7a).

## Discussion

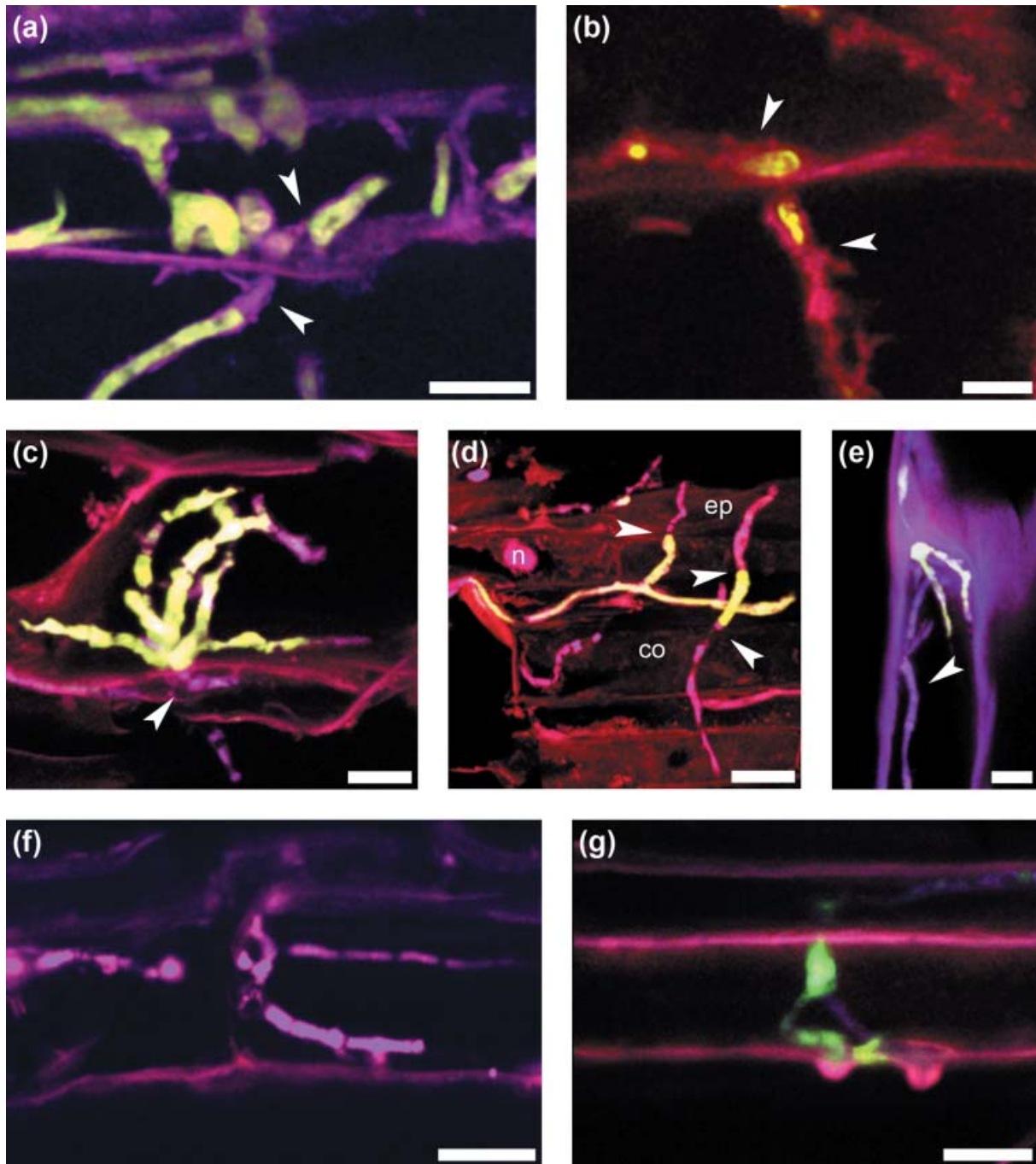
We have applied two new tools for the detection and quantification of *F. equiseti* and *P. chlamydo-sporea* colonizing barley roots. The use of real-time PCR with specific primers and molecular beacon probes allowed us to quantify the extent of the fungal colonization, both overall and in terms of endophytic growth, of barley roots by both fungal species more accurately than culturing methods. This is, to the best of our knowledge, the first application of molecular beacon probes for quantification of fungi within plant tissues, growing either as endophytes or as pathogens (Schena *et al.*, 2004). In spite of the advantages of this type of fluorogenic probe in comparison to other detection methods, such as SYBR Green I or TaqMan probes (Tyagi & Kramer, 1996; Bonnet *et al.*, 1999), these have been more commonly used for detection of plant viruses (Gonçalves *et al.*, 2002) or putative bacterial bioterror agents (Varma-Basil *et al.*, 2004). There is, however, a report of a previous *vcp1*-based molecular beacon for detection of *P. chlamydo-sporea* in soil (Rosso *et al.*, 2007). Quantification of *P. chlamydo-sporea* in soil has been performed in previous works using competitive PCR (Mauchline *et al.*, 2002) and real-time PCR with an



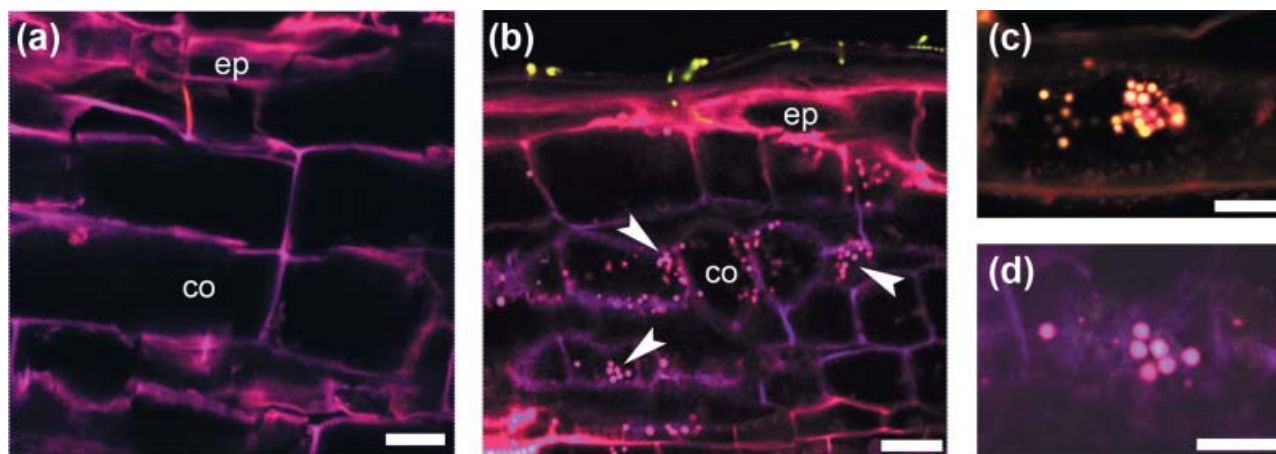
**Fig. 4** Laser scanning confocal microscopy of barley (*Hordeum vulgare*) root colonization by Fe10331gfp. (a) Fe10331gfp transformant showing constitutive expression of the green fluorescent protein (GFP) gene. Note conidiophores (arrowhead) with fluorescent macroconidia. Bar, 50  $\mu$ m. (b) Early colonization of the upper part of the roots at 3 d after inoculation (dai), with formation of an external hyphal network and penetration of the root epidermis by single hyphae (arrowheads). Bar, 50  $\mu$ m. (c) Outer and inner colonization of the upper part of the root at 7 dai, with reduction of the outer hyphal network density. Bar, 50  $\mu$ m. (d) Cryosection of a barley root at 7 dai showing colonization of epidermal and outer cortical cells. co, cortical cell; ep, epidermal cell; rh, root hair. Bar, 20  $\mu$ m. (e) Appressorium-like structure formed by a hypha colonizing adjacent cortical cells at 5 dai. Bar, 5  $\mu$ m. (f) Hyphal swellings appearing intracellularly in a cortical cell at 5 dai. Bar, 5  $\mu$ m. (g) Single cortical cell filled with hyphae 5 d after inoculation. Bar, 10  $\mu$ m. (h) Septate hyphal swelling localized intercellularly within the root cortex at 5 dai. Bar, 15  $\mu$ m.



**Fig. 5** Laser scanning confocal microscopy of barley (*Hordeum vulgare*) root colonization by Pc123gfp. (a) Pc123gfp transformant showing constitutive expression of the green fluorescent protein (GFP) gene. Note conidiophores (arrowhead) with fluorescent conidia. Bar, 50  $\mu$ m. (b) Outer colonization of the root at 7 d after inoculation (dai), with formation of a hyphal network. Bar, 50  $\mu$ m. (c) Penetration of root epidermal cell layers by a group of external hyphae at 3 dai. Bar, 20  $\mu$ m. (d) Massive attachment of hyphae to epidermal cell walls with penetration of plant cell lumen at 7 dai. Bar, 10  $\mu$ m. (e) Penetration of a root epidermal cell wall by an appressorium-like structure, prevented by formation of a papilla around it. Bar, 10  $\mu$ m. (f) Intracellular colonization of epidermal root layers at 3 dai. Bar, 20  $\mu$ m. (g) Colonization of the root cortex at 7 dai. Bar, 20  $\mu$ m. (h) Production of chlamydospores (arrowhead) on the root surface at 3 dai. Bar, 20  $\mu$ m. (i) Merged brightfield and 505–530-nm laser line image of a chlamydospore within a cortical cell at 7 dai. Bar, 10  $\mu$ m. co, cortical cell; ep, epidermal cell; rh, root hair.



**Fig. 6** Laser scanning confocal microscopy of barley (*Hordeum vulgare*) roots colonized by either Fe10331gfp or Pc123gfp stained with FM4-64. (a) Plant-derived membrane (arrowheads) surrounding *Fusarium equiseti* hyphae 3 d after inoculation (dai). Bar, 10  $\mu$ m. (b) *Pochonia chlamydosporia* appressorium-like structure forming a penetration hypha between two root cortical cells closely fitted in a plant-derived membrane (arrowheads) at 3 dai. Bar, 5  $\mu$ m. (c) Point of penetration of a cortical root cell by an *F. equiseti* hypha at 5 dai, with generation of multiple intracellular invasive hyphae. Note the loss of viability of the penetrating hypha as shown by the lack of green fluorescent protein (GFP) fluorescence (arrowhead). This image is a two-dimensional maximum projection of 16 optical sections acquired with a z-interval of 1.18  $\mu$ m. Bar, 10  $\mu$ m. (d) Loss of viability of *F. equiseti* hyphae (arrowheads) colonizing root cortex cells at 3 dai, generated from viable GFP-expressing hyphae, observed by the use of differential wavelength readings to detect separately the plant's cell wall autofluorescence and FM4-64. co, cortical cell; ep, epidermal cell; n, plant cell nucleus. This image is a two-dimensional maximum projection of 20 optical sections acquired with a z-interval of 0.49  $\mu$ m. Bar, 20  $\mu$ m. (e) Root cortical cell colonized by both viable and unviable (arrowhead) *P. chlamydosporia* hyphae at 5 dai. Bar, 10  $\mu$ m. (f) Root cortical cells at 7 dai filled with degenerated *F. equiseti* hyphae detected by FM4-64 staining, with complete absence of GFP-expressing hyphae. Bar, 20  $\mu$ m. (g) Papilla formation in a root cortical cell to prevent invasion by *F. equiseti* hyphae between neighbouring cells at 7 dai. Bar, 5  $\mu$ m.



**Fig. 7** Detection of abundant production of fluorescent vacuoles in barley (*Hordeum vulgare*) roots inoculated with either Fe10331gfp or Pc123gfp, observed by laser scanning confocal microscopy and staining with FM4-64. (a) Uninoculated control root with absence of fluorescent vacuoles. Bar, 20  $\mu$ m. (b) Overview of the disposition of vacuoles (arrowheads) in deep cortical cells in a Pc123gfp-colonized root at 7 d after inoculation (dai). Bar, 20  $\mu$ m. (c) Close-up of fluorescent vacuoles within a cortical cell in Fe10331gfp-colonized roots at 7 dai. Bar, 10  $\mu$ m. (d) Close-up of fluorescent vacuoles within a cortical cell in Pc123gfp-colonized roots at 7 dai. Bar, 10  $\mu$ m. co, cortical cell; ep, epidermal cell.

ITS-based Scorpion-PCR probe (Ciancio *et al.*, 2005). No previous work on detection of *F. equiseti* using real-time PCR has been published, although other *Fusarium* species have been quantified on different substrates by the application of TaqMan probes (Gao *et al.*, 2004; Waalmijk *et al.*, 2004; Sarlin *et al.*, 2006).

Real-time PCR quantification data were supported in parallel by the monitoring of spatial and temporal patterns of root colonization using GFP-tagged *F. equiseti* and *P. chlamydosporia* transformants observed by confocal microscopy, which were obtained by genetic transformation mediated by *A. tumefaciens* with two different vectors. For both fungal species, stable transformants were obtained, although the frequency of transformation was low. Optimization of the transformation protocol for each fungus should increase its efficiency, as has been found for other fungal species (Rho *et al.*, 2001; Sugui *et al.*, 2005). To the best of our knowledge, this is also the first application of *A. tumefaciens*-mediated transformation for both *F. equiseti* and *P. chlamydosporia* species. Genetic transformation of *Fusarium* species has commonly been achieved by formation of protoplasts. By contrast, protoplast-mediated *P. chlamydosporia* transformation has encountered difficulties because of the mitotic instability of the transformants (Atkins *et al.*, 2004; López-Serna, 2004).

Real-time PCR assays allowed us to detect amounts of target DNA as low as 1 pg, as reported in other detection studies (Atkins *et al.*, 2003; Gao *et al.*, 2004; Waalmijk *et al.*, 2004; Ciancio *et al.*, 2005; Sarlin *et al.*, 2006). Assays with other related fungal species confirmed the specificity of the primer–probe set for the detection of *F. equiseti*, while in the case of *P. chlamydosporia* complete specificity could not be achieved, because detection of *P. rubescens* (even with a high  $C_t$  value) by real-time PCR was obtained. The alkaline serine

protease P32 (Lopez-Llorca, 1990) gene from *P. rubescens* has not been sequenced to date, in spite of our trials with primers derived from the *P. chlamydosporia* *vcp1* gene. This could indicate significant differences in the gene sequences of the two species. Nevertheless, the target DNA sequence for MBPC was found to be identical for *P. chlamydosporia* and *P. rubescens*, and therefore the low yield in real-time PCR detection of the latter may be attributable to single nucleotide polymorphisms in the primer-binding regions.

DNA extraction from unsterilized and surface-sterilized roots allowed us to compare both overall and endophytic populations of each fungal species colonizing the barley roots. Previous work has shown the ability of sodium hypochlorite to efficiently destroy nucleic acids from different samples (Thornbury & Farman, 2000; Phe *et al.*, 2004). Therefore, sodium hypochlorite-based surface sterilization of roots would denature the DNA of epiphytes while that of endophytes would remain available for amplification. For the two fungi, quantification of the root colonization yielded similar values, and colonization of the root surface was always higher than colonization of internal root tissues. The colonization ratios between surface-sterilized and unsterilized roots found by real-time PCR were generally larger than those obtained using culturing methods. When root colonization was high (e.g. in unsterilized roots) the isolation rate obtained by culturing was saturated (e.g. 100%), perhaps because this method was not sensitive enough to detect differences in fungal biomass (i.e. a root piece containing a single fungal spore and a root piece completely covered by a hyphal network would both be scored as showing positive growth). We conclude that PCR-based techniques are more sensitive than conventional culturing methods, although an increase in the sample size of the latter could make results more comparable.

Spatial analysis of *F. equiseti* and *P. chlamydosporia* within barley roots using laser confocal scanning microscopy revealed the patterns and dynamics of root colonization. Both fungal species formed a dense hyphal network on the root surface, while single hyphae penetrated and colonized epidermal and cortical cells. Nevertheless, colonization patterns varied substantially between *F. equiseti* and *P. chlamydosporia*. Colonization of roots by Fe10331gfp closely followed that observed for the wt *F. equiseti* Fe10331 in Maciá-Vicente *et al.* (2008b); however, further information was obtained as a result of the enhanced contrast between hyphae and root tissues achieved by use of GFP-expressing strains. This allowed an easier detection of hyphal clusters and swellings within host root cells, which could be related to nutrient absorption, and intercellular septate structures resembling chlamydospores. Colonization of barley roots by Pc123gfp was slower than that of *F. equiseti*, probably because of the slower growth rate of the former. Root colonization patterns of the GFP-tagged *P. chlamydosporia* confirmed observations for the wt Pc123 in Bordallo *et al.* (2002). During the first colonization events, *P. chlamydosporia* colonized the root as a single hypha exploring the root surface, usually in regions with an abundance of root hairs (probably because of initial attachment to these by conidia), which developed into a hyphal net surrounding the root. Penetration of the root epidermis occurred mostly through cell walls via the formation of appressorium-like structures, and colonization was predominantly intracellular.

We also found evidence for formation of a membrane sheath by the host plant sealing the penetrating hyphae of the endophytic fungi within root cells. This is similar to the plant-derived extra-invasive hyphal membrane (EIHM) described for the rice invasion events of the blast fungus *Magnaporthe oryzae* (Kankanala *et al.*, 2007). These structures are also well known in arbuscular mycorrhizal (AM) symbioses. The narrow space between the plant membrane and the hyphal cell wall is filled by extracellular matrix material similar in composition to the primary cell wall (Balestrini & Bonfante, 2005). This is an active signalling interface between the host plant and AM fungus to establish the symbiosis at cellular and molecular levels (Genre *et al.*, 2005). In our study, *F. equiseti* and *P. chlamydosporia* did not maintain the integrity of the membrane sheath, as it could not be stained with FM4-64 in the later stages of cell colonization. Barley cell membrane sheaths would prevent symplast invasion in the early stages of colonization, and their later degradation would lead to subsequent penetration of hyphae into the cell lumen.

The endophytic behaviour observed for both *F. equiseti* and *P. chlamydosporia* is probably responsible for the elicitation of the plant defence systems, with formation of abundant papillae within the cells, as previously observed by Bordallo *et al.* (2002), and vacuoles. Autofluorescence of the vacuole content may also indicate the presence of phenolic compounds, which are produced in response to fungal infection

and directly fluoresce upon excitation under the laser confocal microscope (Hutzler *et al.*, 1998). This might also explain the presence in these root regions of degraded or nonfluorescent hyphae within the plant tissues. These hyphae were easily detected using FM4-64 staining. Whether the DNA contents of these hyphae remain undegraded or not will require further investigation, such as the application of *in situ* PCR or reverse transcriptase (RT)-PCR techniques to detect the potential activity of the nucleus in degraded hyphae (Haase *et al.*, 1990; Seddas *et al.*, 2008). Confirmation of hyphal death resulting from the host response, and development of living hyphae in new parts of the root as a way to escape the defence reaction, would provide an example of the balanced antagonism hypothesis for endophytes proposed by Schulz *et al.* (1999). However, fungal DNA may remain undegraded within the root tissues, as has been found for plant DNA (Chiter *et al.*, 2000), and therefore quantification by real-time PCR would lead to false positives. Gamper *et al.* (2008) described a lack of correlation between the real-time PCR quantification of both AM DNA and RNA within the host roots and the real hyphal length measured by microscopy methods. This was attributable to an uneven distribution of nuclei and physiological activities between vital hyphae (which in many cases did not harbour nuclei) and spores, as assessed by dual nuclear and vital staining. In spite of these limitations, real-time PCR is a valuable technique for quantification of total nucleic acid contents of specific fungi within plant tissues, in combination with the microscopy tools applied in this work.

Root endophytic performance by biocontrol fungi is an important factor for control of root pathogens, through phenomena such as escape to competition with soil microbiota, enhancement of plant growth, and modulation of plant defences. In the present work we have developed two different methodologies to study *F. equiseti* and *P. chlamydosporia* as root endophytes and used live-cell imaging to study root colonization. The availability of these tools will allow further studies on the biology of these fungi and on their capacity for biocontrol of root pathogens, in both semi-field and field conditions. These studies are in progress in our laboratory.

## Acknowledgements

The authors thank Laura Rosso and Dr Aurelio Ciancio, Istituto per la Protezione delle Piante, Consiglio Nazionale delle Ricerche, Bari, Italy, for valuable help in molecular beacon probe design, Prof. Michael J. Bidochka, Department of Biological Sciences, Brock University, ON, Canada, for kindly supplying *Agrobacterium tumefaciens* AGL-1 strain containing plasmid pFBENGFP, and Dr Zaira Caracuel-Rios, School of Biosciences, University of Exeter, Exeter, UK, for help in genetic transformation of isolates. This work was supported by CICYT research grants (AGL2007-60264/AGR and AGL2008-00716/AGR) and a scholarship to JGMV (AP2002-093) from the Spanish Ministerio de Ciencia e Innovación.

## References

- Atkins SD, Clark IM, Sosnowska D, Hirsch PR, Kerry BR. 2003. Detection and quantification of *Plectosphaerella cucumerina*, a potential biological control agent of potato cyst nematodes, by using conventional PCR, real-time PCR, selective media and baiting. *Applied and Environmental Microbiology* 69: 4788–4793.
- Atkins SD, Mauchline TH, Kerry BR, Hirsch PR. 2004. Development of a transformation system for the nematophagous fungus *Pochonia chlamydosporia*. *Mycological Research* 108: 654–661.
- Ausubel FM, Brent R, Kingstone RE, Moore DD, Seidman JG, Smith JA, Struhl K. 2002. *Short protocols in molecular biology*, Vol. 2, 5th edn. New York, NY, USA: Wiley.
- Balestrini R, Bonfante P. 2005. The interface compartment in arbuscular mycorrhizae: a special type of plant cell wall? *Plant Biosystems* 139: 8–15.
- Bloemberg GV, Camacho-Carvajal MM. 2006. Microbial interactions with plants: a hidden world. In: Schulz B, Boyle C, Sieber T, eds. *Microbial root endophytes*. Heidelberg, Germany: Springer-Verlag, 320–336.
- Bonnet G, Tyagi S, Libchaber A, Kramer FR. 1999. Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proceedings of the National Academy of Sciences, USA* 96: 6171–6176.
- Bordallo JJ, Lopez-Llorca LV, Jansson H-B, Salinas J, Persmark L, Asensio L. 2002. Colonization of plant roots by egg-parasitic and nematode-trapping fungi. *New Phytologist* 154: 491–499.
- Chiter A, Forbes JM, Blair GE. 2000. DNA stability in plant tissues: implications for the possible transfer of genes from genetically modified food. *FEBS Letters* 481: 164–168.
- Ciancio A, Loffredo A, Paradies F, Turturo C, Sialer F. 2005. Detection of *Meloidogyne incognita* and *Pochonia chlamydosporia* by fluorogenic molecular probes. *EPO Bulletin* 35: 157–164.
- Deshmukh S, Hüchelhoven R, Schäfer P, Imani J, Sharma M, Weiss M, Waller F, Kogel K-H. 2006. The root endophytic *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proceedings of the National Academy of Sciences, USA* 103: 18450–18457.
- Fang W, Pei Y, Bidochka MJ. 2006. Transformation of *Metarhizium anisopliae* mediated by *Agrobacterium tumefaciens*. *Canadian Journal of Microbiology* 52: 623–626.
- Gamper HA, Young JPW, Jones DL, Hodge A. 2008. Real-time PCR and microscopy: are the two methods measuring the same unit of arbuscular mycorrhizal fungal abundance? *Fungal Genetics and Biology* 45: 581–596.
- Gao X, Jackson TA, Lambert KN, Li S, Hartman GL, Niblack TL. 2004. Detection and quantification of *Fusarium solani* f. sp. *glycines* in soybean roots with real-time quantitative polymerase chain reaction. *Plant Disease* 88: 1372–1380.
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118.
- Geiser DM, Jiménez-Gascó MM, Kang S, Makalowska I, Veerarahavan N, Ward TJ, Zhang N, Kuldau GA, O'Donnell KO. 2004. FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. *European Journal of Plant Pathology* 110: 473–479.
- Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG. 2005. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. *The Plant Cell* 17: 3489–3499.
- Gonçalves MC, Klerks MM, Verbeek M, Vega J, van den Heuvel JFJM. 2002. The use of molecular beacons combined with NASBA for the sensitive detection of sugarcane yellow leaf virus. *European Journal of Plant Pathology* 108: 401–407.
- de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology* 16: 839–842.
- Haase AT, Retzel EF, Staskus KA. 1990. Amplification and detection of lentiviral DNA inside cells. *Proceedings of the National Academy of Sciences, USA* 87: 4971–4975.
- Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.
- Horinouchi H, Muslim A, Suzuki T, Hyakumachi M. 2007. *Fusarium equiseti* gf191 as an effective biocontrol agent against *Fusarium* crown and root rot tomato in rock wool systems. *Crop Protection* 26: 1514–1523.
- Hortal S, Pera J, Parladé J. 2008. Tracking micorizas and extraradical mycelium of the edible fungus *Lactarius deliciosus* under field competition with *Rhizopogon* spp. *Mycorrhiza* 18: 69–77.
- Hutzler P, Fischbach R, Heller W, Jungblut TP, Reuber S, Schmitz R, Veit M, Weissenböck G, Schnitzler J-P. 1998. Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany* 49: 953–965.
- Jacobs H, Gray SN, Crump DH. 2003. Interactions between nematophagous fungi and consequences for their potential as biological agents for the control of potato cyst nematodes. *Mycological Research* 107: 47–56.
- Kankanala P, Czymbek K, Valent B. 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *The Plant Cell* 19: 706–724.
- Kerry BR. 1993. The use of microbial agents for the biological control of plant-parasitic nematodes. In: Jones DG, ed. *Exploitation of microorganisms*. New York, NY, USA: Chapman & Hall, 81–104.
- Kirk AA. 1993. A fungal pathogen with potential for biocontrol of *Striga hermonthica* (Scrophulariaceae). *BioControl* 38: 459–460.
- Lazo GR, Stein PA, Ludwig RA. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio/Technology* 9: 963–967.
- Lee S, Kim SH, Breuil C. 2002. The use of the green fluorescent protein as a biomarker for sapstain fungi. *Forest Pathology* 32: 153–161.
- Lopez-Llorca LV. 1990. Purification and properties of extracellular proteases produced by the nematophagous fungus *Verticillium subclasperium*. *Canadian Journal of Microbiology* 36: 530–537.
- Lopez-Llorca LV, Bordallo JJ, Salinas J, Monfort E, López ML. 2002. Use of light and scanning electron microscopy to examine colonisation of barley rhizosphere by the nematophagous fungus *Verticillium chlamydosporium*. *Micron* 33: 61–67.
- Lopez-Llorca LV, Duncan JM. 1986. New media for the estimation of fungal infection in eggs of the cereal cyst nematode, *Heterodera avenae* Woll. *Nematologica* 32: 486–490.
- López-Serna ML. 2004. *Interacciones entre hongos patógenos de invertebrados: hongos nematófagos y hongos entomopatógenos*. PhD thesis, University of Alicante, Alicante, Spain.
- Maciá-Vicente JG, Jansson H-B, Abdullah SK, Descals E, Salinas J, Lopez-Llorca LV. 2008a. Fungal root endophytes from natural vegetation in Mediterranean environments with special reference to *Fusarium* spp. *FEMS Microbiology Ecology* 64: 90–115.
- Maciá-Vicente JG, Jansson H-B, Mendgen K, Lopez-Llorca LV. 2008b. Colonization of barley roots by endophytic fungi and their reduction of take-all caused by *Gaeumannomyces graminis* var. *tritici*. *Canadian Journal of Microbiology* 54: 600–609.
- Mauchline TH, Kerry BR, Hirsch PR. 2002. Quantification in soil and rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. *Applied and Environmental Microbiology* 68: 1846–1853.
- Monfort E, Lopez-Llorca LV, Jansson H-B, Salinas J, Park JO, Sivasithamparam K. 2005. Colonisation of seminal roots of wheat and barley by egg-parasitic nematophagous fungi and their effects on *Gaeumannomyces graminis* var. *tritici* and development of root-rot. *Soil Biology & Biochemistry* 37: 1229–1235.
- Morton CO, Hirsch PR, Peberdy JP, Kerry BR. 2003. Cloning of and genetic variation in protease VCP1 from the nematophagous fungus *Pochonia chlamydosporia*. *Mycological Research* 107: 38–46.

- Nitao JK, Meyer SLF, Schmidt WF, Fettinger JC, Chitwood DJ. 2001. Nematode-antagonistic trichothecenes from *Fusarium equiseti*. *Journal of Chemical Ecology* 27: 859–869.
- O'Donnell K, Cigelnik E, Nirenberg HI. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* 90: 465–493.
- Phe MH, Dossot M, Block JC. 2004. Chlorination effect on the fluorescence of nucleic acid staining dyes. *Water Research* 38: 3729–3737.
- Rho H-S, Kang S, Lee Y-H. 2001. *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. *Molecules and Cells* 12: 407–411.
- Rosso LC, Ciancio A, Finetti-Sialer M. 2007. Application of molecular methods for detection of *P. chlamydosporia* from soil. *Nematropica* 37: 1–8.
- Sarlin T, Yli-Mattila T, Jestoi M, Rizzo A, Paavanen-Huhtala S, Haikara A. 2006. Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. *European Journal of Plant Pathology* 114: 371–380.
- Schena L, Nigro F, Ippolito A, Gallitelli D. 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology* 110: 893–908.
- Schulz B, Boyle C. 2005. The endophytic continuum. *Mycological Research* 109: 661–686.
- Schulz B, Rommert AK, Dammann U, Aust HJ, Strack D. 1999. The endophyte-host interaction: a balanced antagonism? *Mycological Research* 103: 1275–1282.
- Seddas PMA, Arnould C, Tollot M, Arias CM, Gianinazzi-Pearson V. 2008. Spatial monitoring of gene activity in extraradical and intraradical developmental stages of arbuscular mycorrhizal fungi by direct fluorescent *in situ* RT-PCR. *Fungal Genetics and Biology* 45: 1155–1165.
- Sesma A, Osbourn E. 2004. The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* 431: 582–586.
- Siddiqui ZA, Akhtar MS. 2008. Synergistic effects of antagonistic fungi and a plant growth promoting rhizobacterium, an arbuscular mycorrhizal fungus, or composted cow manure on populations of *Meloidogyne incognita* and growth of tomato. *Biocontrol Science and Technology* 18: 279–290.
- Sugui JA, Chang YC, Kwon-Chung KJ. 2005. *Agrobacterium tumefaciens*-mediated transformation of *Aspergillus fumigatus*: an efficient tool for insertional mutagenesis and targeted gene disruption. *Applied and Environmental Microbiology* 71: 1798–1802.
- Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673–4680.
- Thornbury DW, Farman ML. 2000. Reusing nylon-membranes for radioactive hybridizations. *BioTechniques* 29: 1250–1254.
- Tyagi S, Kramer FR. 1996. Molecular beacons: probes that fluoresce upon hybridisation. *Nature Biotechnology* 14: 303–308.
- Varma-Basil M, El-Hajj H, Marras SAE, Hazbon MH, Mann JM, Connell ND, Kramer FR, Alland D. 2004. Molecular beacons for multiplex detection of four bacterial bioterrorism agents. *Clinical Chemistry* 50: 1060–1063.
- Waalwijk C, van der Heide R, de Vries I, van der Lee T, Schoen C, Costrel-de Corainville G, Kastelein P, Köhl J, Lonnet P, Demarquet T *et al.* 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. *European Journal of Plant Pathology* 110: 481–494.
- White TM, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal DNA for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego, CA, USA: Academic Press, 315–321.
- Winton LM, Stone JK, Watrud LS, Hansen EM. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. *Phytopathology* 92: 112–116.